Antibiotics Stimulate Formation of Vesicles in Staphylococcus aureus in both Phage-Dependent and -Independent Fashions and via Different Routes

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Abstract: Bacterial membrane vesicles research has so far mainly focussed on Gram-negative bacteria. Only recently Gram-positive bacteria have been demonstrated to produce and release extracellular membrane vesicles (MVs) that contribute to bacterial virulence. Although treatment of bacteria with antibiotics is a well-established trigger of bacterial MVs formation, the underlying mechanisms are poorly understood. In this study we show that antibiotics can induce MVs through different routes in the important human pathogen DNA damaging agents and antibiotics inducing the SOS response triggered vesicle formation in lysogenic strains of but not in their phage-devoid counterparts. -lactam antibiotics flucloxacillin and ceftaroline increased vesicle formation in a prophage-independent manner by weakening the peptidoglycan layer. We present evidence that the amount of DNA associated with MVs formed by phage lysis is higher than that of MVs formed by -lactam antibiotics-induced blebbing. The purified MVs derived from protected the bacteria from challenge with daptomycin, a membrane-targeting antibiotic, both and in whole blood. In addition, the MVs protected from killing in whole blood, indicating that antibiotic-induced MVs function as a decoy and thereby contribute to the survival of the bacterium.

DOI: https://doi.org/10.1128/AAC.01439-18

Antibiotics stimulate vesicles formation in *Staphylococcus aureus* in a phage-dependent and independent fashion and via different routes

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Running Head: Membrane vesicles induction in *Staphylococcus aureus*

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ABSTRACT

Bacterial membrane vesicles research has so far mainly focussed on Gram-negative bacteria. Only recently Gram-positive bacteria have been demonstrated to produce and release extracellular membrane vesicles (MVs) that contribute to bacterial virulence. Although treatment of bacteria with antibiotics is a well-established trigger of bacterial MVs formation, the underlying mechanisms are poorly understood. In this study we show that antibiotics can induce MVs through different routes in the important human pathogen *Staphylococcus aureus*. DNA damaging agents and antibiotics inducing the SOS response triggered vesicle formation in lysogenic strains of *S. aureus* but not in their phage-devoid counterparts. β-lactam antibiotics flucloxacillin and ceftaroline increased vesicle formation in a prophage-independent manner by weakening the peptidoglycan layer. We present evidence that the amount of DNA associated with MVs formed by phage lysis is higher than that of MVs formed by β-lactam antibiotics-induced blebbing. The purified MVs derived from *S. aureus* protected the bacteria from challenge with daptomycin, a membrane-targeting antibiotic, both *in vitro* and *ex vivo* in whole blood. In addition, the MVs protected *S. aureus* from killing in whole blood, indicating that antibiotic-induced MVs function as a decoy and thereby contribute to the survival of the bacterium.

INTRODUCTION

Bacterial membrane vesicles (MVs) affect diverse biological processes, including virulence, DNA transfer, export of cellular metabolites, phage decoy and cell-to-cell communication, as shown in various bacterial species (1-3). Although MVs were originally shown to originate from blebbing of the outer membrane of Gram-negative bacteria, evidence has accumulated that various Gram-positive bacteria also release MVs composed of cytoplasmic membrane (2). A recent study has shown that MVs formation in the Gram-positive bacterium *Bacillus subtilis* is stimulated by the expression of an endolysin, which is encoded by a defective prophage (4). The hydrolytic activity of the endolysin creates small holes in the peptidoglycan layer through which cytoplasmic membrane (CM) material can protrude and is released as cytoplasmic MVs (CMVs). It has been observed that these cells lose CM integrity as indicated by the formation of ghost cells containing many intracellular CMVs. Therefore, this vesicle biogenesis mechanism has been named “bubbling cell death” (Masanori Toyofuku, Nobuhiko Nomura, Leo Eberl “Types and origins of bacterial membrane vesicles” Nature Reviews Microbiology, doi:10.1038/s41579-018-0112-2, in press).

Prophages are integrated in the bacterial host genome and are passed on to daughter cells at each cell division. When the host cell experiences genotoxic stress, e.g. by exposure to DNA damaging agents or UV radiation, the prophage induces the expression of lytic genes that promote DNA replication, phage particle assembly, DNA packaging, and eventually bacterial lysis to release the new phage particles along with vesicles that are also formed in this process (4). Another route that stimulates CMVs formation through the weakening of the cell wall is the treatment with β-lactam antibiotics (4-6), which leads to protrusion of the cytoplasmic membrane into the extracellular space. While it is well known that MVs

formation is stimulated by antibiotic treatment, the underlying mechanisms are only poorly understood.

Staphylococcus aureus (*S. aureus*) causes a wide spectrum of human infections, ranging from superficial cutaneous infections to systemic infections, including pneumonia, osteomyelitis, endocarditis, and bacteremia (7). The production of CMVs plays an important role in the delivery of *S. aureus* virulence factors into eukaryotic host cells (8, 9), stimulates biofilm formation (10), increases the resistance to killing by whole blood and activates purified human neutrophils *ex vivo.* Mice previously immunized with CMVs were protected against subcutaneous and systemic *S. aureus* infection (11). CMVs were also shown to increase antibiotic resistance, as they can contain β-lactamases (12). Here we used lysogenic *S. aureus* strains and their phage-devoid counterparts to investigate the effect of different antibiotics on CMVs formation. Our results show that antibiotics can induce vesiculation by at least two routes, in a phage-dependent as well as phage-independent fashion, depending on the mode of action of the antibiotic compound. We further examined how the different routes resulting in CMVs production influence the content of the CMVs as well as their efficacy in protecting against antibiotic killing.

RESULTS

To test whether temperate phages affect vesicle formation in *S. aureus* we compared the amounts of CMVs produced by the MSSA clinical strains NRS135 and RN4220 and their phage-bearing counterparts NRS77^{phage} and RN4220^{phage}, respectively (Tab. 1). Under standard growth conditions, no significant difference in CMVs production was observed (Fig. 1). However, upon treatment with bacterial growth subinhibitory concentrations of the DNA damaging agent mitomycin C (MMC), a well characterized trigger of the SOS response (13), a

strong increase in CMVs formation was observed in the strains carrying a prophage but not in the cured strains devoid of phages (Fig. 1A). Moreover, CMVs formation was stimulated in a concentration-dependent manner in the lysogenic strains (Fig. 1B). Using transmission electron microscopy we observed CMVs, phages and ghost cells, indicative of endolysin-triggered cell lysis, in samples of the MMC-treated, phage-carrying strains (Fig. 2A, C and E). Ghost cells were rarely observed in untreated controls (Fig. 2F) or cured strains. These data suggest that phage-triggered cell lysis is an important mechanism of CMVs formation in *S. aureus*.

As some antibiotics, particularly quinolones, are known to induce the cellular SOS response (14, 15), we tested next whether treatment with ¼ or 10 times the minimal inhibitory concentration (MIC) of ciprofloxacin (CIP) would affect CMVs production. In the lysogenic strain NRS77^{phage} but not in the phage-free background strain NRS135 we observed a clear increase in vesicles production (Fig. 3A and C). With strain RN4220 we observed a similar trend at MIC ¼, while the differences were not statistically significant for MIC10 (Fig. 3B and D).

Another possibility to stimulate vesicle formation in Gram-positive bacteria is to inhibit cell wall biosynthesis with β-lactam antibiotics (4-6). Treatment with β-lactam antibiotics is thought to weaken the peptidoglycan such that cytoplasmic membrane material can protrude into the extracellular space and is released as CMVs. Exposing the *S. aureus* strains to 10 times the MIC of the β-lactam antibiotics flucloxacillin (FLU) or ceftaroline (CPT) strongly increased vesicle formation independent of the presence or absence of the prophage (Fig. 3C and D). When exposed to ¼ the MIC of the antibiotics, a clear, phage-independent effect was observed for CPT but not for FLU, which at this low concentration caused a modest increase of vesiculation only in the lysogenic strain NRS77^{phage} (Fig. 3A and

114 B). While TEM micrographs showed large amounts of CMVs, phages could not be observed
115 when bacteria were treated with 10 times the MIC of FLU (Fig. 2B), indicating that these
116 vesicles do not originate from SOS-induced bubbling cell death but likely through an
117 alternative blebbing mechanism (Fig. 2D).
118 To investigate whether the cargo carried by CMVs originating from blebbing or SOS-induced
119 cell lysis are different, we determined the amount of DNA associated with the CMVs (Fig.
120 4A). These data provided clear evidence that CMVs originating from phage lysis have a
121 generally higher DNA content than CMVs originating from a blebbing mechanism.
122 We next tested whether *S. aureus* CMVs can protect cells from the last-resort antibiotic
123 daptomycin (DAP), which inhibits cell envelope synthesis by interfering with fluid membrane
124 microdomains (16). Due to its intrinsic resistance to infection with the phages produced by
125 RN4220^{phage} and NRS77^{phage} strains, the clinical *S. aureus* strain CI1449, a bloodstream
126 isolate recovered from a patient with *S. aureus* endocarditis, was chosen. Incubation of the
127 CI1449 strain with a phage lysate obtained from the NRS77^{phage} strain did not result in phage
128 binding to the bacterial surface (Fig. S1C and D), while binding of phages was observed for
129 strain NRS135 (Fig. S1A and B), indicating that strain CI1449 lacks a functional phage
130 receptor. This allowed us to test the protective effect of MMC-induced CMVs as they co-
131 purified with phages (Fig. 2A), which would lyse sensitive strains. We assessed DAP killing in
132 the presence or absence of purified CMVs both *in vitro* as well as *ex vivo* using freshly-drawn
133 human whole blood.
134 For the *in vitro* experiments exponentially growing phage-resistant *S. aureus* strain CI1449
135 ($5*10^7$ CFUs, high inoculum; and 10^5 CFUs, low inoculum) were challenged with two
136 different concentrations of CMVs. FLU-induced CMVs exhibited a clear concentration-
137 dependent protective effect against DAP as early as 30 minutes after the start of the assay

for both inocula size tested (Fig. 5C and D). Although slightly reduced, a protective effect was also observed for MMC-induced CMVs (Fig. 5A and B). For the ex *vivo* experiments $2.3*10^4$ *S. aureus* CI1449 cells were suspended in whole blood and CMVs and DAP were added (either separately or after pre-incubation for 30 minutes in whole blood). Pre-incubation resulted in an enhanced protective effect against DAP challenge, which was observed as early as 15 minutes after the start of the assay for both MMC and FLU-induced CMVs (Fig. 6A and B). Without pre-incubation the protective effect was slightly reduced.

We also observed a significant protective effect of CMVs against whole blood killing for both MMC and FLU-induced CMVs after 120 minutes of incubation, confirming that CMVs can also protect against the host's innate immune system (Fig. 6C and D). In agreement with the *in vitro* data, the level of protection conferred by MMC-induced CMVs *ex vivo* was lower than by FLU-induced CMVs.

DISCUSSION

This study demonstrates that treatment of *S. aureus* with the DNA damaging agent MMC or specific antibiotics routinely used in clinics increases CMVs production both in a phage-dependent as well as phage-independent fashion, depending on the mode of action of the compounds. We provide evidence that at least two different mechanisms account for this stimulatory effect: MMC and CIP induce the SOS response and consequently increase vesicle formation through endolysin-triggered cell death, provided the strain harbors a temperate phage. On the other hand, the β-lactams FLU and CPT, at 10 times MIC, weaken the cell wall and thereby stimulate CMVs production through a phage-independent blebbing mechanism. A significant induction of vesiculation was observed already at ¼ MIC of CPT, while this low concentration of FLU only weakly increased vesicle formation in the lysogenic strain

NRS77^{phage}. No difference in CMVs production was observed for untreated strains, independently of the presence of prophages. This is in agreement with a recent study showing that under non-inducing growth conditions, phage-encoded endolysins are not essential for CMVs formation which, however, depends on the host's autolysin Sle1 (13, 17). We also observed a difference in the DNA cargo harbored by CMVs produced by strains NRS135 and NRS77^{phage} after stimulation with the DNA damaging agents MMC and CIP. This difference possibly occurs because the phage lytic cycle involves the degradation of the host chromosome resulting in smaller DNA fragments that may be more efficiently packed into vesicles. This idea is supported by a recent report showing that treatment of *Stenotrophomonas maltophilia* with CIP not only stimulates the production of high content DNA MVs but also of large numbers of phages, which are both a consequence of SOS response induction in this bacterium (18). In contrast, no significant difference in DNA load was observed between lysogenic and cured strains for CMVs produced by induction with the cell membrane-damaging agents FLU and CPT.

Previous work has shown that CMVs can provide protection against host defence factors such as antimicrobial peptides from mammalian tissue and complement system factors of the blood (19-21). In addition, it was recently shown that vesicles can serve as decoys for phages and membrane-targeting antibiotics (22, 23). In agreement with this study, we show that both FLU- and MMC-induced CMVs protect cells from the membrane-active antibiotic DAP not only *in vitro* but also *ex vivo* in whole blood. This result suggests that the protective effect is relevant in a more physiological condition, which may more closely reflect the situation of an infected patient. The lower degree of protection we observed for MMC-induced CMVs in the bacterial protection assay, as compared to FLU-induced CMVs, could be due to the fact that CMVs induced by the SOS-response activation carry phage endolysins

that can affect cell viability (4, 24, 25). Askarian et al. recently showed that CMVs naturally produced by *S. aureus* protect cells from whole blood killing (11). We observed a similar effect for CMVs produced by *S. aureus* in response to DNA damage or antibiotic stress, suggesting that the mechanism of CMV genesis does not affect the protective function of CMVs against the host innate immune defence (11). As phage binding to the surface of strain CI1449 is impaired, the presence of phages in MMC-induced CMVs preparations is not responsible for the protective effect observed towards DAP and whole blood killing.

The setup we used in this study aimed to reflect the clinical situation. *S. aureus* infections due to methicillin susceptible *S. aureus* are typically treated with FLU alone or in combination with a fluoroquinolone such as CIP, which leads to CMVs release as shown in our study. DAP is used for treating multi-resistant *S. aureus* strains infections, including MRSA strains. In addition, DAP may be added to FLU in case of persisting bacteremia. Our data show that antibiotic-induced CMVs could hinder the action of DAP against *S. aureus* and thus may counteract the clearance of the infection. Interestingly, a recent study showed that DAP can trigger the release of membrane vesicles in *S. aureus*, which may protect the bacterial cells (26). Further work will be required to unravel the differences between DAP- and FLU-triggered CMVs that account for their varied DAP binding affinities.

MATERIAL AND METHODS

Strains and media

S. aureus strains (Tab.1) were grown in LB Lennox (BD) shaking (220 rpm) at 37°C in the presence of MMC (10, 50, 100 or 150 ng/ml) or DAP (Cubicin), CIP (Ciproxin), FLU

(Floxapen), CPT (Zinforo) at an MIC of ¼, 2.3 or 10, depending on the assay (for MIC values see Tab.1).

The RN4220 strain was derived from strain NCTC8325-4 by UV and chemical mutagenesis (27, 28). Its prophage-positive counterpart, strain RN4220^{phage}, was created by phage transduction using *S. aureus* MRSA bacteremia strain 300-169 (29) as prophage donor. To obtain phage preparations, cultures of strain 300-169 were treated with mitomycin C at a concentration of 0.5 µg/ml, as previously described (30). The filtered supernatants corresponding to putative lysates were stored at −80°C before utilization. Phages were propagated using the receiver isolate RN4220. Selection was performed by PCR as prophages are devoid of any resistance markers (Tab. 2). Titrated phage preparations were kept at -80°C. The induced phage preparations were centrifuged on a sucrose density gradient. Phage particles were negatively stained with 2% uranyl acetate, examined in a JEOL 1230 transmission electron microscope at an accelerating voltage of 120 kV, and photographed (31). Morphological types were defined on the basis of phage tail length. The NRS77 strain (depicted as NRS77^{phage} in this work) was cured from phages to give rise to strain NRS135 (32, 33). CI1449 is an invasive *S. aureus* strain isolated at the University Hospital Zurich. It was chosen for bacterial protection and whole blood killing assays due to the fact that it is a blood stream isolate as well as its resistance to infection by the phages harbored in NRS77^{phage} and RN4220^{phage} strains, as confirmed by plaque assay. MIC values were determined using the broth microdilution test in LB medium, as previously described (34).

CMVs induction and quantification

232 *S. aureus* was grown overnight (O/N) in 10 ml of LB Lennox in a 50 ml tube with shaking at
233 220 rpm, diluted in 10 ml of fresh medium in a 50 ml tube to an OD_{600nm} of 0.1 and then
234 cultured in the presence or absence of MMC for 4h or antibiotics for 6h. CMVs were isolated
235 and quantified as previously described (35). Briefly, cell cultures were centrifuged for 10 min
236 at 4,600 g and the supernatant was filtered through a 0.22 µm pore size filter and
237 ultracentrifuged for 1h at 150,000 g at 4°C. The resulting pellets were resuspended in
238 double distilled water for CMVs quantification by staining with the FM1-43 fluorescent dye
239 (Life Technologies, USA). Fluorescence was evaluated by using a Varioscan flash fluorimeter
240 (Thermo Scientifc) or a Synergy HT plate reader (MWG Biotech). Protein content of CMVs
241 preparations was assessed using the BCA protein assay kit (Thermo Scientific). In our
242 system, a value of 1,500 AU corresponds to a CMVs preparation of 250 µg protein/ml.
243
244 **Transmission electron microscopy**
245 CMVs were isolated from bacterial cultures stimulated with either 100 ng/ml MMC or MIC
246 10 of FLU as described above. The isolated CMVs were absorbed on glow-discharged
247 Formvar-coated 300-mesh copper grids and negatively stained with 1% uranyl acetate for
248 visualisation. Bacteria thin sections were prepared as previously described (36) from
249 bacterial cultures stimulated with either 100 ng/ml MMC or MIC 10 of FLU.
250
251 **DNA content measurement**
252 The DNA load of CMVs derived from NRS135 and $NRS77^{phage}$ strains stimulated with either
253 100 ng/ml MMC or MIC 10 of antibiotics was quantified as previously described (37) with
254 some minor modifications. Briefly, CMVs were resuspended in PBS and incubated for 1h in
255 the presence of DNase I (NEB) to degrade extravesicular DNA. The DNase was then

256 inactivated at 75C° for 15 min. DNA was released from CMVs by lysis, using 0.125% Triton X-
257 100. The quantification of DNA was carried out using the PicoGreen dsDNA kit (Invitrogen)
258 and was normalized against CMVs concentrations.
259
260 **Bacterial protection assay**
261 CMVs derived from either MMC (100 ng/ml) or FLU (MIC 10)-stimulated NRS77 bacteria
262 were incubated with DAP (MIC 10) for 2h in LB Lennox at a final concentration of 62.5 or
263 31.25 µg protein/ml. A CI1449 O/N culture was diluted 1:10 in fresh LB and grown for 2h,
264 diluted to an OD_{600nm} of 0.2 in LB+$CaCl_2$ (50 µg/ml final concentration) and added at $5*10^7$
265 (high inoculum) or 10^5 (low inoculum) colony forming units (CFUs)/ml to the CMVs-DAP mix.
266 CFUs counts were determined at 30 min, 1h, 2h, 3h, 4h, 6h and 24h.
267
268 **Whole blood killing**
269 To assess the role of CMVs in protecting bacteria from whole blood killing and DAP
270 challenge in whole blood we used two experimental setups. CMVs and DAP where either
271 pre-incubated in 400 µl of freshly-drawn blood for 30 minutes at 37°C prior to addition of
272 bacteria (DAP+CMVs-pre) or added to 400 µl of freshly-drawn blood at the same time as the
273 bacteria (DAP+CMVs). The CMVs used for this assay were derived from NRS77 cultures,
274 which were either stimulated with MMC (100 ng/ml) or FLU (MIC 10). CMVs were used at a
275 final concentration of 14.4 ug protein/ml and DAP was used at an MIC of 2.3.
276 CI1449 strain was prepared as described for the bacterial protection and added to whole
277 blood to a concentration of $2.3*10^4$ CFUs/ml. Bacterial survival was assessed at 15, 30, 60
278 and 120 minutes by spotting bacterial suspensions of serial dilutions on TSB agar plates.

Bacteria were enumerated after O/N growth at 37°C. Blood was drawn in heparin tubes (BD).

Phage lysates

Lysates of phages carried by the NRS77 strain were prepared as follows. 100 µl of a CMV sample obtained by stimulation of the NRS77 strain with 100ng/ml MMC were added to 300 µl of an O/N culture of the recipient strain NRS135 after addition of 5mM $CaCl_2$ to the growth medium. Following incubation for 15 minutes at room temperature, 3 ml of warm LB soft agar (LB + 0.6% agar) was added and samples were spread on 5% sheep blood plates (Biomerieux). The plates were incubated O/N at 37°C and the phage lysate was harvested in 2 ml LB + 5mM $CaCl_2$ and stored at 4°C after filtration through a 0.45 µm filter.

Phage binding assay

100 µl of phage lysates were incubated with300 µl of an O/N culture of the recipient strains NRS135 and CI1449, after the addition of 5mM $CaCl_2$ to the growth medium. Bacteria were incubated for 15 minutes at room temperature and subsequently washed once in PBS and resuspended in 2.5% glutaraldehyde in 0.1 mM cacodylate buffer. Samples were processed for electron microscopy as described above.

298 **Study approval**

299 Collection of healthy volunteers' blood complied with the current version of the Declaration
300 of Helsinki. The national legal and regulatory requirements and sample collection was
301 approved by the Canton Ethics Committee (Kantonale Ethikkommission Zurich, Switzerland,
302 KEK---2010---0126).

303 **ACKNOWLEDGEMENTS**

304 This work was supported thanks to grants to L.E. (Swiss National Foundation (SNF) Grant
305 #31003A_169307), A.Z. (Swiss National Foundation (SNF) Grant #31003A_176252) and M.T.
306 (JSPS KAKENHI Grant #16H06189 and JST ERATO Grant #JPMJER1502). Imaging was
307 performed with the support of the Center for Microscopy and Image Analysis, University of
308 Zurich. We would moreover like to thank Dr. Kati Seidl for her helpful advice.

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430

431 **FIGURE LEGENDS**

432

433 **Fig. 1. MMC treatment induces CMVs formation in lysogenic *S. aureus* strains NRS and**
434 **RN4220.** Mitomycin C (MMC) was added at 100 ng/ml (**A**) and at the concentrations
435 indicated (**B**) to log phase bacterial cultures of strains NRS135 and RN4220, as well as their
436 lysogenic variants NRS77^{phage} and RN4220^{phage}. After 4 hours of incubation, the amount of
437 CMVs in the supernatants was measured using a fluorescent dye and quantified in arbitrary
438 units (AU). In our system, a value of 1,500 AU corresponds to a CMVs preparation containing
439 250 ug protein/ml. The graphs show data from at least three independent experiments.
440 Statistical analysis was carried out using the unpaired t-test. *=p<0.05; **=p<0.01;
441 ***=p<0.001; ****=p<0.0001.

442

443 **Fig. 2. TEM images of membrane vesicles and *S. aureus* after stimulation with MMC or**
444 **FLU.** CMVs and cells TEM micrographs of *S. aureus* RN4220^{phage} and NRS77^{phage} treated with
445 either 100ng/ml MMC (**A, C, E**) or 10 times the MIC of FLU (**B, D**). Phages are indicated by
446 dashed arrows. In samples of MMC-treated cultures the presence of ghost cells (**C**)
447 (depicted by arrows and enlargement on upper right panel) and of phages (**E**) was observed.
448 (**D**) In cultures treated with FLU, a high number of ghost cells and cells with blebs (indicated
449 by arrows and in the enlargement on upper right panel) were observed. (**F**) Untreated
450 RN4220^{phage} cells.

451

452 **Fig. 3**. **Antibiotics triggered phage-dependent and independent CMVs induction in *S.***
453 ***aureus* NRS and RN4220 strains.** Log phase cultures of strains NRS135 and RN4220 as well
454 as their lysogenic variants NRS77^{phage} and RN4220^{phage} were treated with ciprofloxacin (CIP),

flucloxacillin (FLU) and ceftaroline (CPT) at an MIC of ¼ (**A**, **B**) or at an MIC of 10 (**C**, **D**). The amount of CMVs in the supernatants was measured after 6 hours induction using a fluorescent dye and quantified in arbitrary units (AU). In our system, a value of 1,500 AU corresponds to a CMVs preparation containing 250 ug protein/ml. The graphs show data from at least three independent experiments. Statistical analysis was carried out using the unpaired t-test. *=p<0.05; **=p<0.01; ***=p<0.001; ****=p<0.0001.

Fig. 4. Amounts of DNA associated with CMVs. (**A**) The DNA content of CMVs isolated from *S. aureus* NRS135 and NRS77^{phage} cultures treated with either 100 ng/ml MMC or MIC 10 of the indicated antibiotics was determined. The graphs show data from at least three independent experiments. Statistical analysis was carried out using the unpaired t-test; ***=p<0.001; ns=not significant.

Fig. 5. CMVs derived from *S. aureus* NRS77^{phage} stimulated with MMC or FLU protect a clinical *S. aureus* strain from DAP treatment. Log phase cultures of the clinical *S. aureus* isolate CI1449 were treated with 10 times the MIC of DAP in the presence or absence of two different concentrations of CMVs (62.5 or 31.25 µg protein/ml) derived from *S. aureus* NRS77^{phage} stimulated with (**A**, **B**) MMC (100 ng/ml) or (**C**, **D**) FLU (MIC 10). Viability of the *S. aureus* clinical isolate CI1449 was followed over 24 hours. Strain CI1449 is resistant to infection by phages produced by strain NRS77^{phage} upon treatment with MMC. High inoculum: 5*10^{7} CFUs/ml; low inoculum: 10^{5} CFUs/ml. Missing data points indicate no detectable live bacteria; the lower detection limit for this assay is 10^{3} CFUs/ml. The graphs show data from at least three independent experiments.

Fig. 6. CMVs derived from *S. aureus* NRS77^{phage} stimulated with MMC or FLU protect a clinical *S. aureus* strain from daptomycin killing in whole blood and from whole blood killing. 2.3*10^4 CFUs/ml of the clinical *S. aureus* isolate CI1449 were incubated in 400 µl blood in the presence or absence of DAP (MIC 10) and/or CMVs (14.4 µg protein/ml) derived from either MMC (**A and C**) or FLU (**B and D**). Data from at least three independent experiments are shown. Statistical analysis was carried out using the unpaired t-test. Results of statistical analysis are indicated on the graphs. *=p<0.05; **=p<0.01; ***=p<0.001; ****=p<0.0001; ns=not significant.

488 **TABLES**

489

		MIC (ug/ml)				
***S. aureus* strain**	**Reference**	**MMC**	**DAP**	**CIP**	**FLU**	**CPT**
RN4220^{phage}	(27-29) P. François laboratory	0.125	4	0.50	0.0625	4
RN4220	(27, 28)	0.500	2	0.25	0.0625	4
NRS77^{phage}	(32, 33)	0.250	4	0.25	0.0625	4
NRS135	(32, 33)	0.500	4	0.50	0.1250	4
CI1449	A. Zinkernagel laboratory	NA	2	NA	NA	NA

490

491 **Table 1 – *S. aureus* strains and MICs -** All strains were grown in LB Lennox broth (BD) at 37°C
492 aerobically under constant shaking at 220 rpm or plated on Tryptic Soy Broth (TSB) agar
493 (BD). MICs were determined in LB by broth microdilution test (34). MMC = mitomycin C;
494 DAP = daptomycin; CIP = ciprofloxacin; FLU = flucloxacillin; CPT = ceftaroline

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Primer name	**Sequence**

F1_phageKati	TCCATTGCATGTTGTCACCT
R1_phageKati	ATTTCAGCGGCTTGTTTTGT
F2_phageKati	TAAATTGGTGCGTCAGCTTG
R2_phageKati	ATCAGCATTTGATGGCGTTT
SAM_F35	ATGACCCATGGGAAGCATAT
SAM_R1124	GTTTGTGCATATGACGCTCA

502

503 **Table 2 – Primers used for the selection of the RN4220^{phage} strain –** The primers were used

504 to screen for the presence of prophages in the RN4220 strain genome.

Figure 1

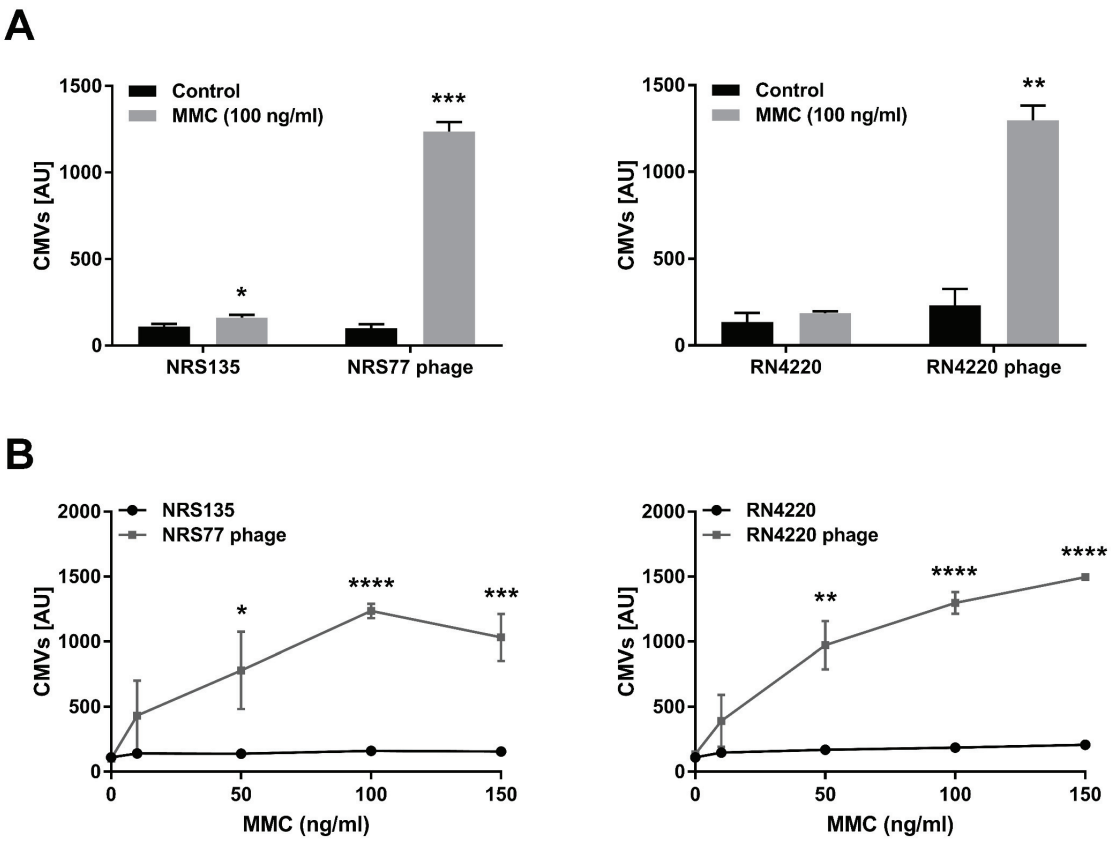

Figure 2

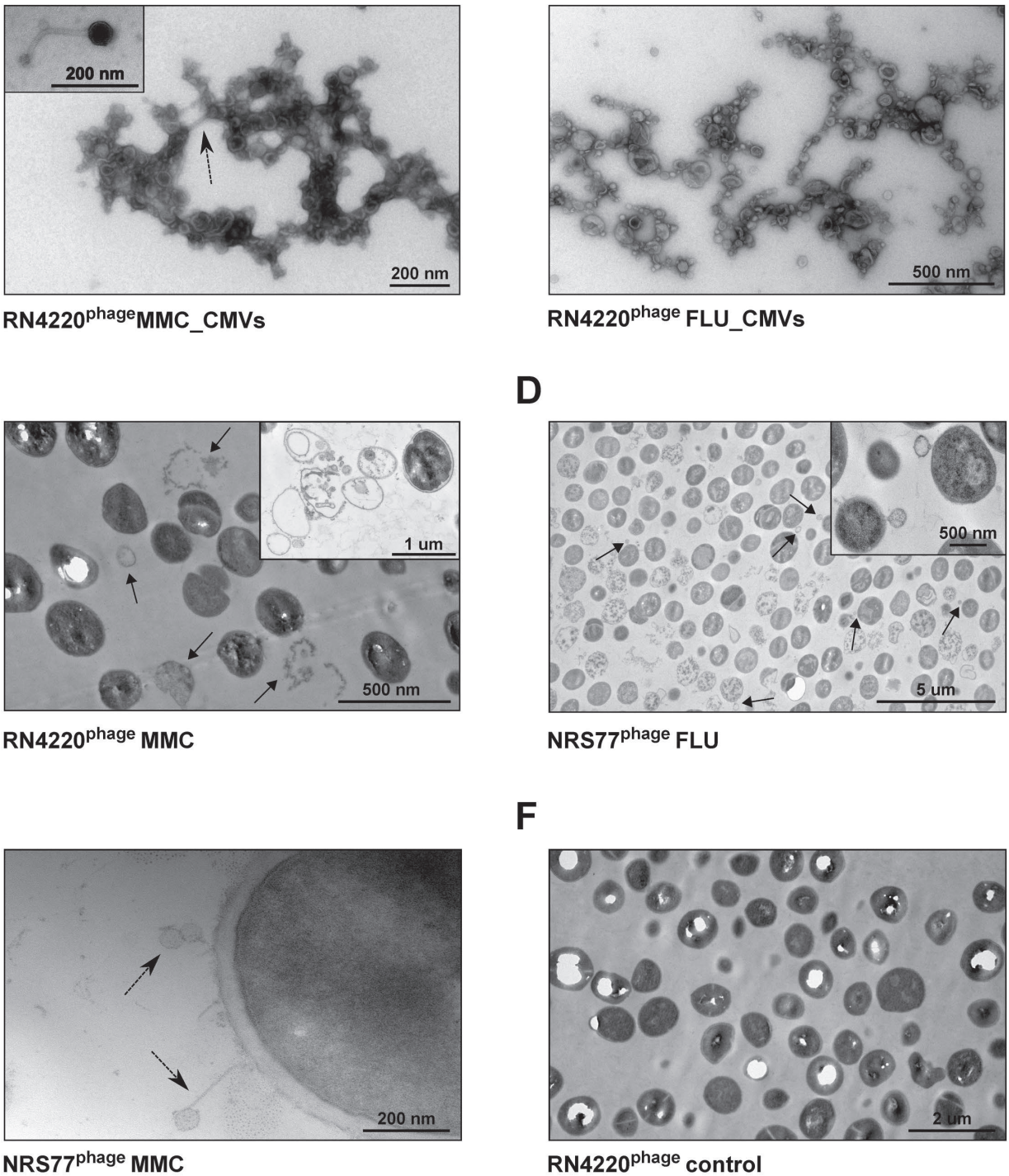

Figure 3

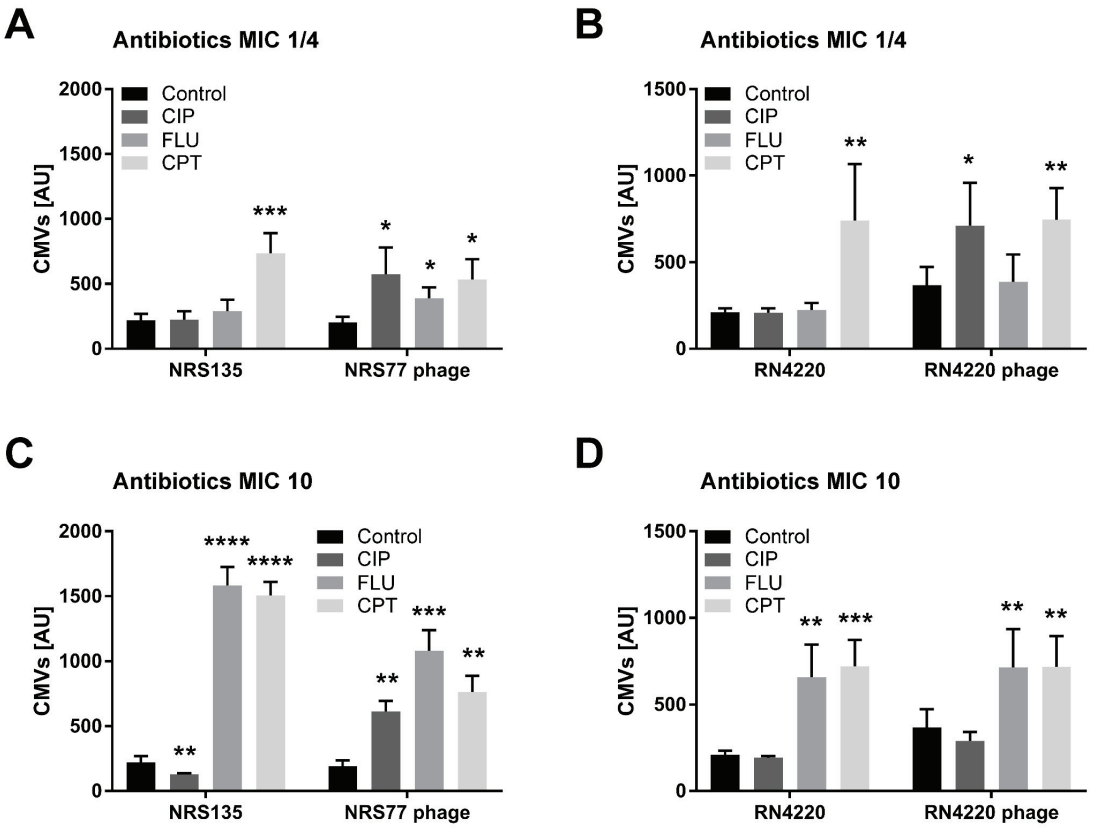

Figure 4

A

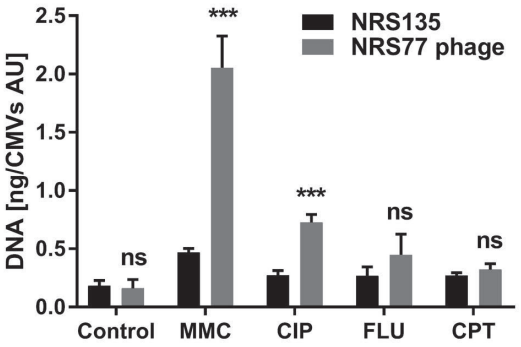

Figure 5

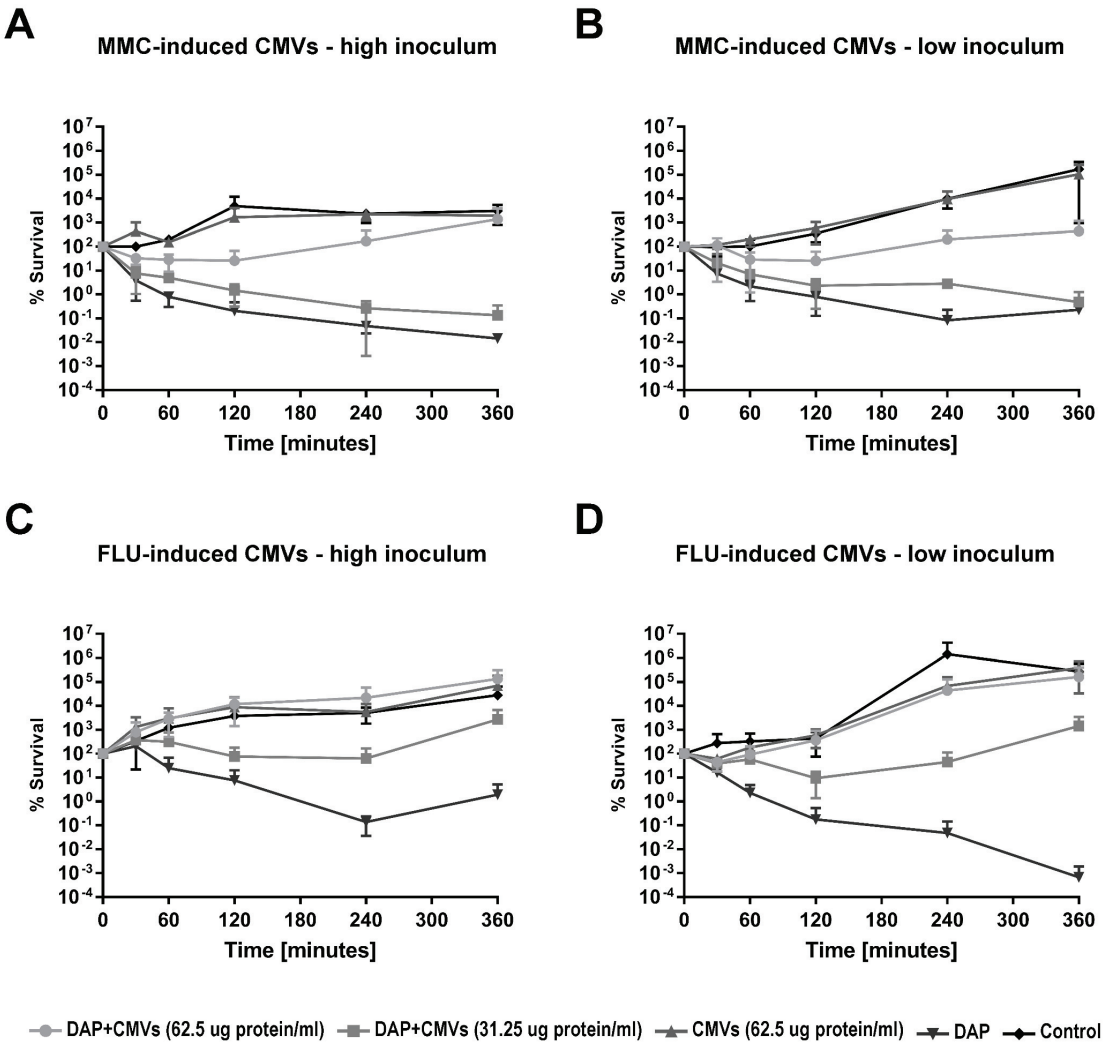

Figure 6